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Effects of Methylprednisolone on Human Myeloid Leukemic Cells In Vitro

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We have demonstrated previously that high-dose methylprednisolone treatment induces differentiation and apoptosis of leukemic cells in patients with different morphological subtypes of acute myeloblastic leukemia (AML) in vivo. In the present study, we investigated the in vitro effects of high (10^{-3} M) and low (10^{-6} M) concentrations of methylprednisolone (MP) on freshly isolated bone marrow leukemic cells from nine newly diagnosed patients with AML by light and electron microscopy (EM) and agarose gel electrophoresis. A marked increase in MP-induced apoptosis of leukemic cells, with a maximum effect at 24 hr of exposure to both low and high concentrations of MP (10^{-6} M and 10^{-3} M), was demonstrated by light microscopy in cultures of four (three with AML-M1 and one with AML-M7) of the nine patients. In three cases, the increase in the number of apoptotic cells induced by high-concentration MP was approximately twice that observed when the lower concentration was used. A few apoptotic cells were detected in the cultures from the other five patients. However, a typical DNA ladder pattern of apoptosis was observed on gel electrophoresis of MP-treated leukemic cells from one patient (AML-M1) after 2 hr of incubation with both high- and low-MP concentrations. In two patients, a nonspecific DNA smear was observed only when high-concentration MP was used. The increase in differentiated leukemic cells induced by MP was also dose dependent, and was observed in cultures from all but one patient. Morphological features of apoptosis and differentiation were also confirmed by EM studies. The results of the present study, together with our previous clinical experience, suggest that MP, especially at high doses, could have a significant role in the treatment of some AML patients by inducing apoptosis and differentiation of leukemic cells. Am. J. Hematol. 60:255–259, 1999.

Key words: apoptosis; acute myeloblastic leukemia; children; differentiation; methylprednisolone

INTRODUCTION

Apoptosis, or "programmed cell death," is considered a physiologic mechanism of cell death and differs from necrosis by morphology and biochemistry [1]. Suppression of apoptosis has been implicated as an important pathophysiological event that gives rise to malignant disorders. Therefore, many investigators have attempted to find a therapeutic approach that would restore the physiologic mechanism of cell death in immortal cancer cells.

Glucocorticoids (GCs), that are used extensively in lymphoid malignancies, have been shown to induce cell death by apoptosis [2]. It is generally believed that conventional doses of GCs have limited or no effect in patients with acute myeloblastic leukemia (AML) [3]. However, in addition to several experimental studies that have demonstrated terminal differentiation of mouse myeloid leukemic cells induced by GCs [4,5], high-dose methylprednisolone (HDMP) (20–30 mg/kg/day) has been shown to induce differentiation of myeloid leukemic cells in vivo [6–9]. The latter has had remarkable antileukemic effects in patients with various morphological

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subtypes of AML, as reported by Hiçsönmez et al. [6–9]. Recently, we have also demonstrated in vivo morphologic evidence of apoptosis of differentiated myeloid leukemic cells in children with AML-M3 and AML-M4 following HDMP treatment [10]. However, we could not exclude the possibility of a direct apoptotic effect induced by HDMP at the blast cell level. The aim of the present study was to evaluate the in vitro effects of different concentrations (10^{-3} M and 10^{-6} M) of methylprednisolone (MP) on myeloid leukemic cells obtained from newly diagnosed children with AML.

MATERIAL AND METHODS

Fresh bone marrow (BM) leukemic cells were obtained from nine newly diagnosed children with AML (patient FAB subtypes; five M1, two M2, one M3, and one M7). The samples were obtained before initiation of chemotherapy, and were immediately fractionated on Ficoll gradient, washed in RPMI 1640, and resuspended in the same medium containing 10% fetal calf serum, 100 units of penicillin per ml and 0.1 mg streptomycin per ml. Cells were incubated at 37°C in an atmosphere of 5% CO_2, 95% humidity, and were collected during the exponential growth phase. An improved Neubauer hemocytometer was used to confirm whether adequate numbers of cells had been obtained. The pure form of MP (6 α-MP 21-hemisuccinate) was dissolved in sterile distilled water (Sigma Chemical Co, St. Louis, MO, catalog no. M3781). Thereafter, the cells were cultured with 10^{-3} M and 10^{-6} M MP for up to 48 hr.

The morphological features of apoptosis and differentiation of myeloid leukemic cells were evaluated under light, fluorescent, and electron microscopy (EM). Samples were obtained from cultures before (0) and 2, 6, 12, 24, and 48 hr after exposure to 10^{-3} M and 10^{-6} M MP. The slides obtained from all samples were fixed in methanol and stained with Wright stain for light microscopy. Hoechst 33258 stain, a DNA binding dye, was used to assess apoptosis and differentiation under fluorescent microscopy with an ultraviolet (u.v.)-absorbing barrier filter. Numbers of apoptotic and differentiated cells were determined with an Axioscope (Zeiss, Germany). At least 200 cells were counted on Wright-stained slides obtained from BM cultures. Apoptotic cells were identified by their characteristic condensed chromatin, fragmented nuclei, and apoptotic bodies. Morphological changes suggesting differentiation of leukemic cells toward mature granulocytes were also noted. For EM studies, the cells were washed with phosphate buffered saline and were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) at room temperature for 2 hr. They were then treated with conventional EM techniques. After being embedded in Araldite 6005, thin sections were prepared using an LKB Bromma 8800 ultratome. The cells were stained with lead citrate and uranyl acetate, and were examined in a Jeol 100 CX II (100kV) EM.

To investigate DNA fragmentation, samples were obtained from the cultured BM cells at the same time intervals as those used for the microscopic studies. DNA extraction was performed using the previously described method of Panayiotidis et al. [11]. DNA ladders were demonstrated by agarose gel electrophoresis and viewed by transillumination under u.v. light (302 nm). In addition, before initiation of treatment, p53 expression was investigated in myeloid leukemic cells obtained from the BM cultures of five patients (cases 5 to 9) using the FISH method with a biotin-labeled p53 DNA probe (Oncor, p53 Cosmid/Chromosome 17 a-satellite D17Z1).

RESULTS

Light microscopic examination revealed a marked increase in apoptotic cells and a decrease in blast cells, starting after 2 hr of BM leukemic cell exposure to high and low concentrations of MP (10^{-3}M and 10^{-6}M) in four (cases 1, 5, 6, and 7) of the nine AML patients. The morphologic features of apoptosis consisting of chromatin condensation, reduction of cell volume, and fragmentation of nucleus were detected (Fig. 1). In addition, ultrastructural findings of apoptosis, consisting of margination of nuclear chromatin, dilatation of endoplasmic reticulum cisternae under the cell membrane, vacuolization, and membrane-bound protrusions of the cytoplasm, were also seen (Fig. 2). An increase in the numbers of apoptotic and differentiated cells was observed in cultures after exposure to both concentrations of MP, with a maximum effect observed at 24 hr (Table I). The increase in apoptotic cells induced by high concentration of MP (10^{-3}M) in three patients (cases 5, 6, and 7) was approximately twice that observed at the low MP concentration (10^{-6}M). A few apoptotic cells were detected in the other five patients (cases 2, 3, 4, 8, and 9). Two of these (cases 8 and 9) had p53 mutation. A typical DNA ladder pattern of apoptosis was seen on gel electrophoresis in one patient (case 1), starting after 2 hr of incubation with both MP concentrations. Nonspecific DNA smears were detected in two patients (cases 5 and 6) after 6 hr of incubation of leukemic cells with high-concentration MP.

In all samples except one (from case 8), beginning at 2 hr of incubation, there was an increase in the number of differentiated cells seen in both concentrations of MP. Examination by light and EM revealed morphologic evidence of differentiation of leukemic cells consisting predominantly of metamyelocytes and granulocytes (Figs. 1 and 2). The increase in the number of differentiated cells was found to be greater after exposure to 10^{-3} M MP only. BM cells obtained from case 4 (AML-M3) revealed a five-fold increase in the number of differentiated cells

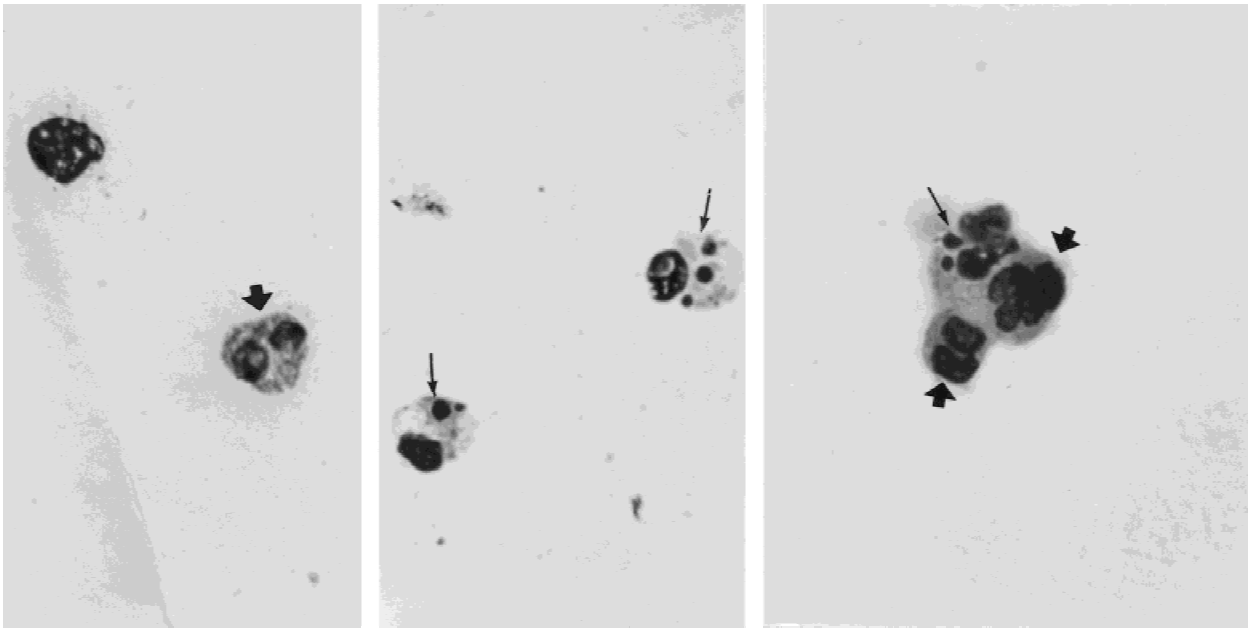

Fig. 1. Apoptotic (↑) and differentiated (➧) cells after addition of 10^{-3} M and 10^{-6} M MP to myeloid leukemic cells (magnification, ×250—Wright stain).

TABLE I. In Vitro Effects of MP on Myeloid Leukemic Cells*

				Apoptotic Cells (%)[a]			Differentiated Cells (%)[a]			Blasts (%)[a]		
					After MP			After MP			After MP	
Cases	FAB	Age/sex	p53 status	Pretreatment	10^{-3} M[b]	10^{-6} M[b]	Pretreatment	10^{-3} M[b]	10^{-6} M[b]	Pretreatment	10^{-3} M[b]	10^{-6} M[b]
1	M1	16/F	NS	0	74	68	6	14	6	92	12	26
2	M2	9/F	NS	0	9	4	2	16	7	98	75	89
3	M1	6/M	NS	0	6	4	1	8	5	99	86	91
4	M3	14/F	NS	0	3	4	4	25	5	96	72	91
5	M1	15/F	WT	0	24	14	3	14	10	97	62	76
6	M1	15/M	WT	1	23	12	4	14	8	95	63	80
7	M7	9/M	WT	0	25	12	3	12	8	97	63	81
8	M1	12/M	M	0	4	0	6	8	6	94	88	93
9	M2	7/M	M	0	4	3	7	20	10	93	76	90

*MP, methylprednisolone; WT, wild type p53; M, mutant p53; NS, not studied.
[a]Percent of apoptotic, differentiated, and blast cells obtained 24 hr after addition of MP.
[b]MP concentration in culture.

after 24 hr of incubation with 10^{-3} M MP (Table I). The nuclear findings of apoptosis and differentiation were also confirmed by Hoechst stain. Interestingly, after 6 hr of incubation in 10^{-3} M MP, platelet-producing cells were detected on light microscopic examination of a BM sample from a case with AML-M7 (Fig. 3).

DISCUSSION

Morphological changes resembling apoptosis in cultures of human lymphoblastoid cell lines exposed to low (10^{-6}M) and high (10^{-3}M) doses of steroid were described by Blewitt et al. [12]. These investigators obtained a marked cytolethal response with high-dose steroid in all the cell lines they tested. In contrast, this effect was observed with low-dose steroid only in a sensitive lymphoid cell line. A massive lethal effect was also demonstrated in human myeloid leukemic cells exposed to a suprapharmacologic dose of MP (10^{-3} M) by Bird et al. [13]. More recently, dexamethasone-induced apoptosis has been demonstrated in an AML cell line with a t(8;21) chromosome translocation [14].

We have shown that addition of both low (10^{-6} M) and high (10^{-3} M) concentrations of MP to myeloid leukemic

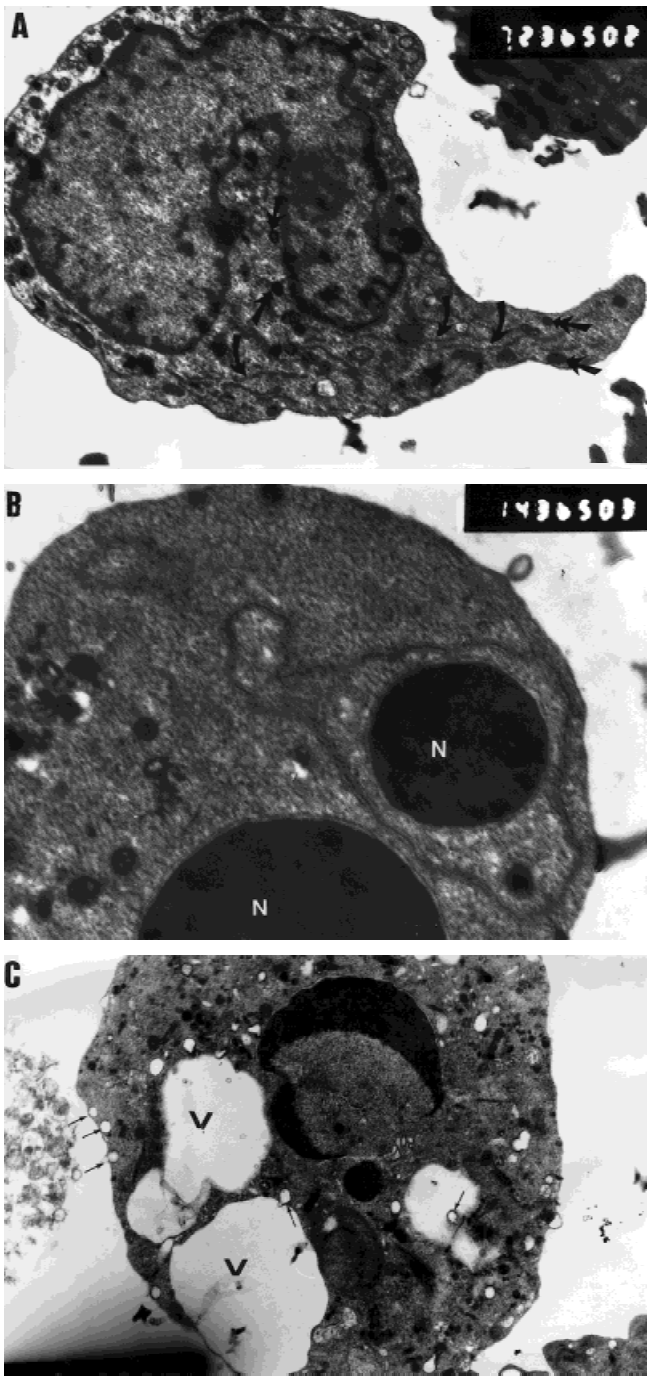

Fig. 2. Electron micrographs of case 1 (A) differentiated cell with indented nucleus and well-developed granular endoplasmic reticulum (GER) (↑) and azurophil granules (▲) (magnification, ×7,200) and (B) apoptotic cell with condensed fragmented nuclear pieces (N) 12 hr after addition of 10^{-3} M MP. Note irregular cell surface with villous projections (magnification, ×14,000). (C) condensation and margination of chromatin in nucleus. In the cytoplasm, big vacuoles (V) and dilated cisternae of GER (↑) under the plasmalemma and adjacent to big vacuoles (V) are noticed after 24 hr incubation with 10^{-3} M MP (magnification, ×7,200).

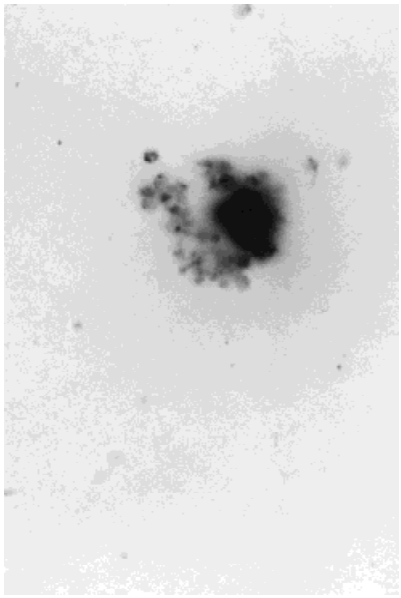

Fig. 3. Platelet producing cells 6 hr after addition of 10^{-3} M MP (case 7 with AML-M7) (Wright stain, magnification ×250).

cells from four (three AML-M1, one AML-M7) of nine AML patients resulted in a dose-dependent, marked increase in apoptotic cell numbers (Table I). However, only a few apoptotic cells were detected in the cultures of samples from the other five patients, two of whom had p53 mutation. The tumor suppressor gene wild-type p53 has been shown to be involved in the control of apoptosis in some murine myeloid leukemic cells [15]. Mutant p53 suppressed the apoptosis by diminishing the effects of deregulated c-myc [16]. The expression of mutant p53 in human myeloid leukemic cells may be associated with suppression of MP-induced apoptosis. Further studies are required to clarify the relationship between the expression of p53 mutation and MP-induced apoptosis in patients with AML.

In the present study, a DNA ladder pattern was demonstrated in both concentrations of MP-treated BM cells in only one patient (case 1) in whom the highest number of apoptotic cells was detected after exposure to MP. Although DNA fragmentation is considered a characteristic feature of apoptosis, apoptotic cell death with characteristic cytoplasmic morphologic features can also occur in enucleated cells [17]. In addition, Ormerod et al. [18] showed that DNA can be cut into larger (30 kbp) rather than smaller (180 bp) fragments, resulting in DNA smear instead of a DNA ladder on gel electrophoresis. This could be the reason for the DNA smearing observed in two of our patients. Ormerod et al. suggested that the DNA fragmentation might be characteristic but not necessary for identifying apoptosis, and that microscopy should always be carried out to support the diagnosis of apoptosis. Apoptosis has been shown in vitro to be the common mode of cell death in differentiated human my-

eloid leukemia cell lines (HL-60 and P39) induced by all-*trans* retinoic acid (ATRA) [19,20]. However, the addition of ATRA to cell cultures obtained from fresh BM cells of 9 AML-M3 patients did not result in apoptosis [21]. In contrast, treatment with HDMP has been shown to induce apoptosis of leukemic cells in vivo in children with AML [10] and myelodysplastic syndrome [22].

An increase in the number of differentiated leukemic cells began 2 hr after the addition of both concentrations of MP in all patients except case 8. The difference in the observed increase in differentiated cells associated with the high vs. the low concentration of MP was not as remarkable as that for the apoptotic cells. This could be attributed to the disappearance of differentiated cells that have already undergone apoptosis. However, samples obtained from an AML-M3 patient (case 4) showed a five-fold increase in differentiated cells in high-dose compared with low-dose MP. Interestingly, in one patient with AML-M7 (case 7), platelet budding from megakaryocytes was detected after 6 hr of incubation in the high concentration of MP only. Further studies are needed to evaluate the effect of MP on the maturation of leukemic cells in patients with AML-M7.

CONCLUSIONS

This study demonstrates that high and low concentrations of MP can induce apoptosis and differentiation of human myeloid leukemic cells in vitro. Greater numbers of apoptotic and differentiated cells were associated with the incubation of high-dose MP. These results point to the importance of clinical use of HDMP in patients with AML.

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